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(57) Abstract

This invention relates to the control of pests. In particular the invention relates to the protection of plants against parasitic nematodes. The invention provides nucleic acid comprising a transcription initiation region capable of directing expression predominantly in the roots of a plant, and a sequence which encodes an anti-nematode protein.

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ROOT SPECIFIC PROMOTERS

This invention relates to the control of pests. In particular the invention relates to the protection of plants against parasitic nematodes.

Nematodes cause global crop losses that have been valued at over $100 billion per year. Examples of particularly important species include *Meloidogyne incognita* and *M. javanica* (a wide range of crops), *Globodera spp* (potato cyst nematodes) *Heterodera schachtii* (beet cyst nematode) and *Heterodera glycines* (soybean cyst-nematode). In addition to direct feeding damage, some nematodes are involved in disease associations. In particular, the *Dorylaimid* nematodes, (*Trichodorus, Paratrichodorus, Longidorus, Paralongidorus and Xiphinema*) transmit NEPO and TOBRA viruses.

The majority of plant parasitic nematodes attack plant roots rather than aerial tissues. Examples of root parasitic nematodes are species of the genera *Heterodera, Globodera, Meloidogyne, Hoplolaimus, Helicotylenchus, Rotylenchoides, Belonolaimus, Paratylenchus, Paratylenchoides, Radopholus, Hirschmanniella, Naccobus, Rotylenchulus, Tylenchulus, Hemicycliophora, Criconemoides, Criconemella, Paratylenchus, Trichodorus, Paratrichodorus, Longidorus, Paralongidorus, Rhadinaphelenchus, Tylenchorhynchus, Hemicriconemoides, Scutellonema, Dolichodorus, Gracilacus, Cacopaurus, Xiphinema* and *Thecavermiculatus*. Host ranges of these species include many of the world's crops and are defined elsewhere (Luc *et al, Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*, CAB International, Wallingford, p629 (1990), Evans *et al, Plant Parasitic*

Nematodes in Subtropical and Tropical Agriculture, CAB International, Wallingford, p648 (1993)).

Root-parasitising nematodes may be ecto- or endo-parasites. In many examples the mouth stylet is inserted and cell contents are removed. Several economically important groups of root parasites have females with a prolonged sedentary phase during which they modify plant cells into nematode feeding sites. Nematodes are the principal animal parasites of plants. They are not herbivores in that they do not ingest whole cells and plant cell walls as characterises the feeding of herbivores such as many insects, molluscs and mammals. The different host-parasite relationships of root feeding nematodes are summarised by Sijmons *et al* (*Annual Review of Phytopathology* **32**: 235-59 (1994)). The requirements for control are therefore distinct from those of other pests such as insects.

This invention has application to any transformable or potentially transformable crop whose root system is damaged by nematodes. This includes a wide range of temperate and tropical crops. The temperate crops to which root parasitic nematodes cause economic damage include: potato, sugar beet, vegetables, oil seed crops, grain, legumes, cereals, grasses, forage crops, forest trees, fruit trees, nut trees, soft fruits, vines, ornamental and bulb crops. Information on the nematode genera and species damaging each of these is given in Evans (1993, *supra*).

A wide range of crops also suffer economic loss from nematodes in tropical and subtropical agriculture. These include: rice (growing in all its cropping ecosystems),

cereals, root and fibre crops, food legumes, vegetables, peanut, citrus, fruit trees, coconut and other palms, coffee, tea and cocoa, bananas, plantains, abaca, sugar cane, tobacco, pineapple, cotton, other tropical fibre crops, and spices. Details of the economic genera and the damage they cause are provided by Luc *et al* (1990, *supra*).

Control of nematodes currently relies on three principal approaches, chemicals, cultural practices and resistant varieties, often used in an integrated manner (Hague and Gowen, *Principles and Practice of Nematode Control in Crops* (Brown, R. H. and Kerry, B. R., eds.), pp. 131-178, Academic Press (1987)). Chemical control is not only costly in the developing world but involves application of compounds including carbamates, such as Aldicarb, which is one of the most toxic and environmentally hazardous pesticides in widespread use. Toxicological problems and environmental damage caused by nematicides has resulted in either their withdrawal or severely restricted their use. They are the most toxicologically and environmentally unacceptable pesticides in widespread use posing considerable risk to aquatic ecosystems and drinking water supplies (Gustafson, *D I Pesticides in Drinking Water, N. Carolina, USA*, p241 (1993)).

Cultural practices such as crop rotation are widely used but they are rarely adequate alone. Resistant cultivars have been a commercial success for a limited range of crops but the approach is unable to control many nematode problems for a variety of reasons (Roberts, *Journal of Nematology*, **24**:213-227 (1992)).

Resistance of crops to nematodes is clearly an important

goal. For nematodes, resistance is defined by the success or failure of reproduction on a genotype of a host plant species. Dominant, partially dominant and recessive modes of inheritance occur based on one or more plant genes. A gene-for-gene hypothesis has been proposed in some cases with typically a dominant R-gene for resistance being countered by a recessive V-gene for virulence in the nematode. Two examples of resistance introduced by breeders are as follows.

In relation to *Globodera spp*, different sources of resistance occur and allow subdivision of potato cyst nematode populations in Europe into two species, each with a number of pathotypes. The European pathotyping scheme envisages eight pathotypes, but the validity and utility of some of the distinctions it makes have been challenged (Trudgill, *1991 Annual Review of Phytopathology* **29**: 167-192). Pathotypes are defined as forms of one species that differ in reproductive success on defined host plants known to express genes for resistance. Use of resistant cultivars may favour selection of certain pathotypes and also favour species unaffected by effective resistance against other nematodes. The H1 gene conferring resistance to certain pathotypes of *Globodera rostochiensis* provided virtually qualitative resistance against UK populations of this nematode, and is widely used commercially. Within the UK, cv Maris Piper expresses H1 and is a highly successful resistant cultivar. Unfortunately, its widespread use in Britain is correlated with an increased prevalence nationally of *G. pallida* to which it is fully susceptible.

A second example occurs in relation to *Meloidogyne* spp.,

morphologically similar forms or races occur with differential abilities to reproduce on host species. The standard test plants are tobacco (cv NC95) and cotton (cv Deltapine) for the four races of *M. incognita* whereas the two races of *M. arenaria* are differentiated by peanut (cv Florrunner). The single dominant gene in tobacco cv NC95 confers resistance to *M. incognita* races 1 and 3 but its cropping in the USA has increased the prevalence of other root-knot nematodes particularly *M. arenaria*. Most sources of resistance are not effective against more than one species of root-knot nematode with the notable exception of the LMi gene from *Lycopersicum peruvanium* which confers resistance to many species except *M. hapla*. Another limitation of resistance genes identified in tomato, bean and sweet potato is a temperature dependence which renders them ineffective where soil temperature exceeds 28°C.

The limitations of conventional control procedures provide an important opportunity for plant biotechnology to produce effective and durable forms of nematode control. Principal advantages are

(i) an approach to pest control that does not require other changes to agronomic practices;

(ii) a reduction in toxicological and environmental risks associated with chemical control; and

(iii) the provision of effective, appropriate and inexpensive crop protection.

Designs for such novel plant defences can be envisaged that lack environmental, producer or consumer risk while

providing substantial economic benefits for both the developed and developing world.

Plant defences against nematodes are known that are additional to the specific genes for resistance reviewed by Roberts ((1992) *supra*). Pre-formed plant defensive compounds may be particularly effective against initial events such as invasion and feeding by nematodes. Such compounds may be lethal to nematodes or act as semiochemicals causing premature exit from the plant. The secondary metabolites involved have been considered by Huang (*An advanced treatise on Meloidogyne volume 1 Biology & Control*, p 165-174, J.N. Sasser & C.C. Carter (eds), North Carolina. State University graphics(1985) although none of these are proteins.

Proteins with roles in plant defence are divided by Bowles (*Ann. Rev. of Biochem.*, **59**:873-907 (1990)) into three groups:

i) those that directly change the properties of the extracellular matrix;

(ii) proteins that have a known direct biological activity against the pathogen or catalyse the synthesis of antimicrobial products; and

iii) proteins whose appearance can be correlated with defence response but which are of unknown function.

Nematode interactions with roots can result in changes in expression of these classes. For instance, changes in peroxidases occur (group (i) above) (Zacheo, G. and

Bleve-Zacheo, T., *Pathogenesis and Host Specificity in Plant Diseases*, Vol II Eukaryotes, ed. Kohmoto, K. Singh U. S. and Singh, R. P., Elsevier, Oxford, UK, p.407 (1995)). Hammond-Kosack *et al* (*Physiol. Mol. Plant Pathol.*, **35**: 495-506 (1989)) showed that pathogenesis-related proteins are induced in plant leaves when nematodes invade roots (group (ii) above) and the promoter of Wun-1 responds to cyst nematode invasion of roots (group (iii) above) (Atkinson *et al*, *Trends in Biotechnology* **13**: 369-374 (1995)). Changes in gene expression within roots are considered in detail by Sijmons *et al* (1994) and Atkinson *et al* (*supra*).

One of the most basic requirements for engineered resistance against a nematode is a plant transformed with an element (promoter) regulating expression of a coding region for an effector protein that disrupts some aspect of the parasitism. Two principal strategies have been devised to-date for nematode control based on transgenic plants utilising two distinct classes of effectors.

The first approach (type 1) is centred on expressing in plants, proteins that do not impair plant growth and yields, but do have anti-nematode effects. This is the approach relevant to this application. The best characterised to-date are proteinase inhibitors.

An example of such an approach can be found in EP-A-0502730 which discloses the use of proteinase inhibitors, eg cowpea trypsin inhibitor (CpTi) and oryzacystatin, to protect plants from nematode parasitism and reproduction. Transgenic plants which express nucleic acid coding for such proteinase inhibitors are also disclosed. Such transgenic plants will therefore be nematode resistant.

These are natural, defence-related, proteins induced in aerial parts of plants and certain other tissues by wounding and herbivory. While they are induced systemically in the aerial parts of plants by nematode parasitism of roots they are surprisingly not present in roots. Cowpea trypsin inhibitor has some potential against insects when expressed as a transgene (Hilder *et al*, *Nature* **220**: 160-163 (1987)). For those advocating their use in transformed plants, PIs have the particular advantage of already being consumed by humans in many plant foods.

The second approach to nematode control (type 2) is not relevant to the present application. It is based on indirect control of nematodes by preventing stable feeding relationships using a concept that has analogy with the plant cell suicide concept of engineered emasculation in maize. This involves expression of a plant cell lethal sequence under the control of a tapetal cell-specific promoter and destroys the male flower (Mariani *et al*, *Nature* **347**: 737-741 (1990)). This approach has been applied to control of cyst and root-knot nematodes (Gurr *et al*, *Mol. Gen. Genet.* **226**: 361-366 (1991); Opperman *et al* (1994)). It relies on identification of feeding site specific promoters or other bases for limiting plant cell death to the feeding cell of the parasite (see Atkinson *et al* (1995) *supra*). It is the search for such promoters that has underpinned much of the work on nematode-responsive plant genes.

There is a clear distinction between direct control of the nematode with anti-parasitic proteins and indirect control by impairing specific plant cells on which certain nematodes depend. These two strategies require

very different promoters to provide expression patterns in plants of interest.

The approach taken in this application has been to identify promoters of value for a generic defence against a wide range of nematode genera. This is important because many important genera attacking plants such as *Belonolaimus*, *Helicotylenchus*, *Hirschmanniella*, *Paratylenchus*, *Radopholus*, *Xiphinema*, *Trichodorus*, *Paratrichodorus*, *Longidorus*, *Paralongidorus*, *Criconemella*, *Rhadinaphelenchus*, *Tylenchorhynchus*, *Hemicycliophora*, *Hemicriconemoides*, *Hoplolaimus*, *Scutellonema*, *Aorolaimus*, *Dolichodorus*, *Rotylenchus*, *Hemicriconemoides*, *Paratylenchus*, *Gracilacus* and *Cacopaurus* do not induce feeding cells. The need is to define genes that are known to be differentially expressed in roots with little expression elsewhere in the plant and to use the promoters associated with these genes. Such promoters enable the provision of pre-formed defences that have no relationship with any known plant defence against nematodes.

Thus, in a first aspect, the present invention provides nucleic acid comprising a transcription initiation region capable of directing expression predominantly in the roots of a plant, and a sequence which encodes an anti-nematode protein.

Suitably, the transcription initiation region will be a promoter, but the invention also encompasses nucleic acid which comprises only those parts or elements of a promoter required to initiate and control expression. Generally, the nucleic acid of the invention will also include a transcription termination region.

The transcription initiation region can be one which is unresponsive to nematode infection. Alternatively, it can be one which will drive expression throughout the roots of a plant in the absence of any nematode infection, but which exhibits a degree of "up-regulation" at an infected locale once infection of the plant occurs.

In the context of the present application, the term "anti-nematode protein" will include all proteins that have a direct effect on nematodes. Examples of such proteins include collagenases (Hausted *et al*, *Conference on Molecular Biology of Plant Growth and Development, Tucson, Arizona* (1991)) and lectins (see, for example, WO 92/15690 which showed that a pea lectin delayed development of *G. pallida* to some extent when expressed transgenically). Cholesterol oxidase expression in transgenic tobacco plants caused the death of bollweevil larvae (Purcell *et al., Biochem. Biophys. Res. Comm. 196:* 1406-1413, (1993)) and may also be effective against nematodes. Expression of peroxidase or oxidase in plants may defend them against nematodes to which it is lethal (Southey *Laboratory methods for work with plant and soil nematodes Ministry of Agriculture, Fisheries and Food, Reference Book 402 HMSO 202pp 1986)*. Transgenic potato plants expressing the hydrogen peroxidase-generating enzyme glucose oxidase have enhanced resistance to bacterial and fungal pathogens (Wu *et al., Plant Cell,* 7:1357-1368, 1995). It is also known that reduced peroxidase activity in tomato plants is associated with increased susceptibility to *Meloidogyne incognita* (Zacheo *et al., Physiological & Molecular Plant Pathology,* **46**:491-507 (1995)).

Expression of antibodies in plants (Hiatt *et al, Nature*

342: 76-78 (1989); Schots *et al*, *Netherlands Journal of Plant Pathology* **98**: 183-191 (1992) may also provide anti-nematode proteins of interest. Antibodies of potential interest include those raised against nematodes (Atkinson *et al*, *Annals of Applied Biology* **112**: 459-469 (1988) and single chain antibody fragments when used alone or when conjugated to an appropriate toxin (Winter and Milstein, *Nature* **349**: 293-299 (1993). This example has been demonstrated by the expression in plants of antibodies directed against a fungal cutinase (Van Engelen *et al.*, *Plant Molecular Biology* **26**: 1701-1710 (1994)). A toxin of interest alone or conjugated to an antibody can include any toxin of *Bacillus thuringiensis* that is effective against nematodes. One report to date is for the efficacy of an exotoxin only (Devidas and Rchberger, *Plant Soil* **145**: 115-120 (1992).

The term anti-nematode protein also includes, but is not restricted to, proteinase inhibitors against all four classes of proteinases and all members within them (Barrett, A. J., *Protein Degradation in Health and Disease, Ciba Foundation Symposium* **75**: 1-13 (1980)).

Other examples of "anti-nematode proteins" include any protein inhibitor of a nematode digestive enzyme. Plant parasitic nematodes contain several enzymes including proteinases, amylases, glycosidases and cellulases (Lee, *The Physiology of Nematodes Oliver & Boyd pp153* (1965)). Starch depletion occurs in nematode feeding cells and has been attributed to nematode amylase activity (Owens & Novotny, *Phytopathology*, **50**:650, 1960). α-amylase inhibitors expressed in transgenic plants provide resistance to pea weevil larvae (Schroeder *et al.*, *Plant Physiology*, **107**:1233-1239: (1995)) and bruchid beetles

(Shade *et al.*, *Bio/Technology*, **12**:793-796: (1994)).

In general the protein will be one which may have a biological effect on other organisms but preferably has no substantial effect on plants.

In one embodiment of this aspect of the invention, the transcription initiation region includes or is the promoter from the b1-tubulin gene of *Arabidopsis* (TUB-1). Northern blots have shown that the transcript of this gene accumulates predominantly in roots, with low levels of transcription in flowers and barely detectable levels of transcript in leaves (Oppenheimer *et al*, *Gene*, **63**:87-102 (1988)). In another embodiment the transcription initiation region is the promoter from the metallothionein-like gene from *Pisum sativum* ($PsMT_A$) (Evans *et al*, *FEBS Letters*, **262**:29-32 (1990)). The $PsMT_A$ transcript is abundant in roots with less abundant expression elsewhere.

Further embodiments of this aspect of the invention include the transcription initiation regions comprising, or being the RPL16A promoter from *Arabidopsis thaliana* (the RPL16A gene from *A. thaliana* encodes the ribosomal protein, L16, its expression being cell specific) or the ARSK1 promoter from *A. thaliana* (the ARSK1 gene encodes a protein with structural similarities to seine/threonine kinases and is root specific). These two promoters are described in more detail in Examples 6 and 7 and the preceding paragraph thereto. Further embodiments include the promoter of the *A. thaliana* AKTI gene. This gene encodes a putative inwardly-directed potassium channel. The promoter preferentially directs GUS expression in the peripheral cell layers of mature roots (Basset *et al.*,

Plant Molecular Biology, **29** : 947-958 (1995) and Lagarde *et al.*, *The Plant Journal*, **9** : 195-203 (1996). Also included is the promoter of the *Lotus japonicus* LJAS2 gene, a gene encoding a root specific asparagine synthetase. Expression of the gene is root specific, as judged by nothern blot analysis (Waterhouse *et al.*, *Plant Molecular Biology*, **30** : 883-897 (1996).

The present invention also describes, as a separate aspect, the manipulation of a transcription initiation region, especially a promoter, to increase its usefulness. Such manipulation may be used to develop a root-specific promoter. In particular, promoter deletions may be created to identify regions of the promoter which are essential or useful for expression in roots and/or to manipulate a promoter to have greater root specificity. Such promoters may be used in conjunction with, but are not limited to, the other aspects of the invention herein described, specifically for use in predominant expression of an anti-nematode protein in the roots of a plant.

A suitable promoter ($PsMT_A$) manipulated as described above is detailed below and in the Examples. The specificity of the promoter is altered by creating deleted versions (constructs) of the promoter. The deleted versions have altered promoter activity and can be used to describe embodiments of the invention. As will be understood by the person skilled in the art, the technique of manipulation can be applied to any transcription initiation region.

As will be understood by the skilled person, any transcription initiation region which directs expression

of a gene(s) predominantly in the roots of a plant can be used according to the invention.

Promoter tagging has been achieved through random T-DNA-mediated insertion of a promoterless *gus*A gene (Lindsey *et al*, *Transgenic Res.* **2**: 33-47 (1993); Topping *et al*, *Development* **112**: 1009-1019 (1991). This provides transgenic β-glucuronidase activity as a reporter gene that is colorimetrically detectable *in plants* (Jefferson *et al*, *EMBO J.* **227**: 1229-1231 (1987). Screening transformed plants e.g. *Arabidopsis*, allows the identification of any promoter tagged by insertion of the *gus*A gene that provides root-specific expression. This approach has been applied to identify differential gene expression in nematode-induced feeding structures (Goddijn *et al* (1993), Sijmons *et al* (1994) *supra*, and Patent Application No PCT/EP92/02559).

It follows that similar approaches can be used to ensure no down-regulation occurs for a root-specific gene on infection of the transformed plant by nematodes as described in this invention. Once such a promoter is tagged, those practised in the art will be familiar with the techniques of inverse Polymerase Chain Reaction (inverse PCR; Doses *et al*, *Plant Molecular Biology* **17**: 151-153 (1991) which will isolate the region 5' to the inserted promoter. If necessary, this provides a clone for screening a genomic library of the plant species (e.g. *Arabidopsis*) to identify putative promoter regions. Methodology for library screening is given in Sambrook *et al*, *infra* (1989). Insertion of *gus*A under control of the putative promoter into a plant such as *Arabidopsis* provides a positive basis for confirming patterns of reporter (GUS) activity. Confirmation is achieved if the

root-specific, expression occurs in uninfected roots as in the original tagged line. This pattern of expression should not be down-regulated by nematode infection as occurs for several promoters examined to date.

The skilled person will appreciate that it is not a requirement of the present invention based on a type I defence that no expression occurs outside of the root system. Providing expression is predominantly in the root system of healthy roots the nucleic acid of the invention offers the prospect of a preformed defence that is not dependent on a response to nematode invasion of the roots.

In addition, promoter deletion studies (Opperman *et al*, *Science*, **263**:221-223 (1994)) have established that the spatial pattern of expression provided by a promoter can be modified. Therefore unwanted, minor spatial patterns of expression can be eliminated by modification of promoters so that only the pattern of interest remains. Thus, this will allow the possibility of eliminating aerial expression without loss of root expression.

The skilled person will appreciate that identification of suitable transcription initiation regions will be relatively straightforward and can be carried out using techniques well known in the art.

The nucleic acid of the invention can be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and,

preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Vectors not including regulatory sequences are useful as cloning vectors.

Nucleic acid of the invention, eg DNA, can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but recombinant DNA technology forms the method of choice.

In a second aspect, the present invention provides the use of nucleic acid comprising a transcription initiation region capable of directing expression predominantly in the roots of a plant, in the preparation of a nucleic acid construct adapted to express an anti-nematode protein.

In a third aspect, the present invention provides a method of conferring nematode resistance on a plant which comprises the step of transforming the plant with nucleic acid as defined herein.

In a fourth aspect, the present invention provides the use of nucleic acid as defined herein in the preparation of a transgenic plant having nematode resistance.

In a fifth aspect, the present invention provides a plant cell transformed with nucleic acid as defined herein.

In a sixth aspect the present invention provides a plant comprising cells transformed with nucleic acid as defined herein.

The present invention thus provides a novel and advantageous approach to the problem of protecting plants, especially commercially important ones, from nematode infestation. In particular, the invention has the following advantages:

a) In contrast to a constitutive promoter such as CaMV35S the anti-nematode protein is expressed principally in roots and not at high levels in the yield or aerial parts of the plant;

b) This restricted expression offers advantages in overcoming regulatory or environmental criticisms of expression of anti-nematode proteins in aerial parts of plants;

c) The approach has the considerable advantage of defending any plant against more than one nematode species during concurrent or sequential parasitism at one site and for localities with dissimilar nematode problems. For example, protection could be provided for upland rice and maize against infection with *Meloidogyne spp and Paratylenchus spp.*

d) The potential in the previous point extends to control of two nematodes forming distinct feeding cells on one host such as *Meloidogyne spp* and *H. glycines* on soybean, *Meloidogyne spp* and *Globodera spp* on potato and *Meloidogyne, Rotylenchulus* on cotton.

e) A general defence against nematodes has commercial value in eliminating the need to determine the presence of nematodes or to quantify economic species.

f) It offers the plant breeding industry a nematode defence readily introduced to any transformable crop species without extensive modification for different nematodes or plant species.

Thus, the skilled person will appreciate that the present invention provides an effective and generic strategy for preventing nematode infestation.

Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*.

The invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention.

The examples refer to the figures, in which:

FIGURE 1: shows the sequence of the TUB-1 promoter;

FIGURE 2: shows the results of expression of GUS under the control of the TUB-1 promoter in transgenic hairy roots of tomato;

FIGURE 3: shows the sequence of the $PsMT_A$ promoter;

FIGURE 4: shows the results of transgenic *Arabidopsis* roots expressing GUS under the control of the $PsMT_A$ promoter;

FIGURE 5: shows the results of *A. thaliana* transformed with $PsMT_A$: GUS construct and infected with *Heterodera schachtii;*

FIGURE 6: shows the extended sequence of the TUB-1 promoter;

FIGURE 7: shows the sequence of the *A. thaliana* RPL16A promoter region cloned into pBI101, in Example 6;

FIGURE 8: shows the results of *A. thaliana* transformed with the RPL16A : GUS construct and stained for GUS activity;

FIGURE 9: shows the sequence of the *A. thaliana* ARSK1 promoter region cloned into pBI101 (in Example 7).

FIGURE 10: shows the sequence of the $PsMT_A$ promoter region, with the extent of the deleted promoter constructs which have been created.

Example 1: Cloning of the TUB-1 promoter

DNA preparation and manipulation

Plasmid DNA was prepared from *E. coli* and *Agrobacterium* cultures by the alkaline lysis method (Sambrook *et al*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1989)). Plasmid DNA was introduced into *E. coli* cells using the $CaCl_2$ transformation procedure (Sambrook *et al*, (1989) *supra*). Restriction digests and ligation reactions were carried out using the recommendations of the enzyme manufacturers.

DNA fragments were recovered from agarose gels using an

electroelution chamber (IBI) according to the manufacturer's protocol. Oligonucleotides were synthesised on an Applied Biosystems 381A instrument and DNA sequencing of double-stranded plasmid DNA was carried out using an ABI automated sequencer according to the manufacturer's recommendations.

Cloning of the TUB-1 promoter

Genomic DNA was prepared from *Arabidopsis thaliana* according to the method of Dellaporta *et al*, *Plant Mol. Biol. Rep.* 1: 19 (1983). The TUB-1 promoter region was amplified by PCR from the *Arabidopsis* genomic DNA using two oligonucleotide primers with the sequences:

5' ATATTAAGCTTGTTACTGTATTCATTACGC 3'

and

5' ACTATGGATCCGATCGATGAAGATTTTGGG 3'

designed from the published sequence of the TUB-1 upstream region (Oppenheimer *et al*, (1988) *infra*). Restriction enzyme sites HindIII and BamHI were incorporated into the primers to aid cloning of the amplified product. The PCR reaction comprised 7.5ng genomic DNA, 200μM dNTPs, 50pmols of each primer and SuperTaq reaction buffer and enzyme at the concentration recommended by the manufacturer (HT Biotechnology Ltd.). 30 cycles of the amplification reaction were carried out with an annealing temperature of 55°C and a 1 minute extension at 72°C.

The amplified DNA was digested with HindIII and BamHI and

a specific DNA fragment of 560bp was recovered from a 1% agarose gel by electroelution. This was cloned into the plasmid vector pUC19 (Yanisch-Perron *et al*, *Gene*, **33**:103 (1985)) and the sequence of the TUB-1 promoter was verified.

The TUB-1 promoter, the sequence of which is shown in Figure 1, was then introduced into the vector pBI101 (Clontech) as a HindIII/BamHI fragment. The HindIII and BamHI restriction sites introduced with the PCR primers are included in the sequence shown in Figure 1. This vector contains the coding region of β-glucuronidase allowing the production of GUS to be used as a reporter of promoter activity in a transformed plant.

Production of transgenic tomato hairy roots

pBI101 containing the TUB-1 promoter fragment was introduced into *Agrobacterium rhizogenes* strain LBA9402 by electrotransformation according to the method of Wen-jun & Forde, *Nucleic Acids Research*, **17**:8385 (1989)). The bacteria were used to transform *Lycopersicon esculentum* cv. Ailsa Craig by a standard protocol (Tepfer, *Cell*, **37**:959-967 (1984)).

Transgenic roots were cultured on 0.5x Murashige and Skoog basal salts mixture supplemented with Gamborgs B5 vitamins, 3% sucrose (w/v) and 0.2% phytagel(w/v). 100mgl-1 kanamycin was included during initial selection. Transgenic root lines were tested for the production of GUS by staining with X-gluc at a concentration of 1mgml-1 in 100mM phosphate buffer pH7.0 containing 10mM EDTA , 0.1% (v/v) Triton X-100 and 0.5mM each of potassium ferricyanide and potassium ferrocyanide (Jefferson *et al*,

(1987) *supra*; Schrammeijer *et al*, *Plant Cell Reports* 9: 55-60 (1990)). Root sections were incubated in the substrate for 12-16 hours.

Infection of roots with *Globodera pallida* and *Meloidogyne incognita*

The J2 of *Globodera pallida* were obtained from cysts and sterilised extensively before use. The cysts were soaked in running tap water for 2-3 days followed by an overnight soak in 0.1% malachite green at room temperature. Cysts were then rinsed for 8h in running tap water prior to soaking overnight at 4°C in an antibiotic cocktail (8mg ml-1 streptomycin sulphate, 6mg ml-1 penicillin G, 6.13mg ml-1 polymicin B, 5mg ml-1 tetracycline and 1mg ml-1 amphotericin B).

The cysts were then washed in filter sterilised tap water and set to hatch in filter-sterilised potato root diffusate. The cysts were placed on a 30 μm nylon mesh secured over a plastic ring and contained within a jar containing a small amount of the sterile potato root diffusate. The jar was placed at 20°C in the dark. The overnight hatch of J2s was collected and sterilised sequentially for 10 min each with the following antibiotics; 0.1% streptomycin sulphate, 0.1% penicillin G, 0.1% amphotericin B and 0.1% cetyltrimethyl-ammoniumbromide (Cetavlon). The nematodes were pelleted between treatments by brief (10s) microcentrifugation. Following sterilisation, they were washed extensively in filter sterilised tap water prior to use.

Roots of transformed lines were cultured for 4 weeks before 2cm lengths including root tips were transferred

to fresh media. After a further 3-4 days, a 5-10µl aliquot containing approximately 35 *G. pallida* J2 was pipetted onto each actively growing root approximately 1cm from its tip. A 1cm² piece of sterile GFA filter paper was placed over each inoculated area to aid infection and was removed 24h later.

Infective juveniles of *Meloidogyne incognita* were obtained from egg masses taken from the galls of infected tomato roots. The galled roots were harvested and rinsed in tap water to remove excess soil. Egg masses were removed from the roots by hand using a scalpel and sterilised sequentially with 0.1% Penicillin G, 0.1% streptomycin sulphate and 0.1% amphotericin B for 30min each followed by 5min in 0.1% Cetavlon. The egg masses were then washed 5-6 times in sterile tap water before being placed on a 30 µm nylon mesh supported between two plastic rings in a jar containing approximately 5ml of sterile tap water. Hatching occurred at 25°C in the dark. The overnight hatch of juveniles was sterilised as for *G. pallida* and the transgenic roots infected in an identical manner.

Investigation of TUB-1 promoter activity in nematode infected transgenic roots

At 7 day time intervals after infection sections were removed from infected transgenic hairy roots. Equivalent pieces were also removed from non-infected, control roots. The roots were rinsed briefly in distilled water to remove any adhering pieces of agar and then immersed in X-gluc solution as previously described. After overnight staining the roots were placed in 1% (v/v) sodium hypochlorite solution for 2min then rinsed in

water and plunged into boiling acid fuchsin (0.035% (w/v) in 25% (v/v) glacial acetic acid) for 2min to stain the nematodes. Roots were then immediately rinsed in distilled water and incubated at 65°C overnight in acidified glycerol to clear the root tissue.

Stained whole root segments were examined using a light microscope at low magnification (x4 - x25) and infected areas were excised and sectioned to a thickness of 100 μm using a vibrating microtome (Oxford). Sections were then mounted in glycerol and examined under both light- and dark-field using a microscope (Leica DM).

Results

Production of transgenic hairy roots

A number of transgenic roots lines were obtained which became blue upon incubation with X-gluc. Two most consistently highly expressing lines were chosen for the infection experiments.

Figure 2 shows the results of GUS expression under the control of the TUB-1 promoter in transgenic hairy roots of tomato.

All roots were stained for GUS activity with X-gluc. In Figure a), roots infected with *Meliodogyne incognita* show strong GUS expression in galls, 14 days after infection.

In b), strong expression of GUS in a large gall induced by *M. incognita* is shown 28 days after infection.

In c) can be seen a section through a gall caused by *M.*

incognita; the centre of the gall stains intensely for GUS activity. In Figure c), f = nematode feeding cells with particularly high TUB-1 promoter activity.

Effect of nematode infection on TUB-1 promoter activity

Stained non-infected control roots were examined and it was clear that the most intense staining occurred in the root tips and at the sites of initiation of lateral roots. However, staining was apparent along the whole length of the roots.

Roots infected with *M. incognita* showed a similar pattern of staining to uninfected roots. TUB-1 promoter was not down-regulated by nematode invasion. In addition, galled regions were stained more intensely than surrounding regions of root. These galled regions were then sectioned using a vibrating microtome to investigate the expression of the GUS gene within the gall. It was found that GUS was present throughout the section and the staining was particularly intense in the giant cells which make up the root-knot nematode feeding site. This heightened intensity at the site of nematode establishment may reflect the multinucleate nature and high metabolic activity of these cells or it may represent a relative upregulation of the TUB-1 promoter in giant cells.

Roots infected with *G. pallida* had a large amount of necrotic tissue surrounding the sites of infection. These cells were presumably killed by the intracellular migration process and consequently they did not stain intensely. However, undamaged cells continued to express GUS. Sectioning of infected regions showed there to be GUS expression within the syncytium (cyst nematode

feeding cell).

Example 2: Cloning of $PsMT_A$

DNA preparation and manipulation

As for Example 1.

GUS expression directed by the $PsMT_A$ promoter

A DNA fragment containing 816bp of 5' flanking region and the first 7 amino acids of the coding sequence of $PsMT_A$ was amplified by PCR and introduced as a HindIII/BamHI fragment into the vector pBI101.2 (Clontech). The sequence of this region is shown in Figure 3. This resulted in a translational fusion between $PsMT_A$ and GUS.

The construct was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation as for TUB-1. This strain was then used to transform *Arabidopsis thaliana* C24 according to the method of Clarke *et al*, *Plant Molecular Biology Reporter*, **10**:178-189 (1992)).

Transformed *Arabidopsis* was grown on 0.5x Murashige & Skoog media containing 10% sucrose(w/v) and 0.2% phytagel (w/v) and selected with 25mgl-1 kanamycin. Staining of roots with X-gluc was then carried out as for TUB-1 transformed hairy roots.

Infective juveniles of *M. incognita* were prepared as before and inoculated onto root tips of transformed *Arabidopsis* seedlings which were 2-3 weeks old. Approximately 30 juveniles suspended in 2% w/v methyl cellulose were pipetted onto each selected root tip. At

7 day intervals after infection plants were carefully removed from the agar and the root systems rinsed in distilled water prior to staining with X-gluc as described previously. If necessary to visualise the nematodes the roots were then counter-stained with acid fuchsin. Roots were first soaked in 1% sodium hypochlorite for 30s then rinsed well in distilled water prior to immersion in boiling acid fuchsin stain (see Example 1) for 30s. Root tissue was cleared in acidified glycerol as for Example 1.

Results

Figure 4 shows the results of transgenic *Arabidopsis* roots expressing GUS under control of the $PsMT_A$ promoter.

All roots were stained for GUS activity with X-gluc. In a), uninfected roots showed strong expression of GUS throughout the root system.

In b), the root system of a plant infected with *M. incognita* 7 days after infection is shown. The arrow indicates a developing gall.

Uninfected roots of *Arabidopsis* plants transformed with $PsMT_A$ promoter:GUS construct showed expression in the root system with slightly reduced staining in young, lateral root tips. Some expression was also observed in senescing aerial tissue. Plants infected with *M. incognita* still exhibited strong expression throughout the root system with more intense staining of gall tissue surrounding the nematode.

Infective juveniles of *Heterodera schachtii* were obtained

from cysts and sterilised extensively before use. Cysts were incubated in 0.1 % malachite green for 30 minutes at room temperature and rinsed in running tap water for 1 h prior to soaking overnight at 4 °C in an antibiotic cocktail containing 8 mg ml^{-1} streptomycin sulphate, 6 mg ml^{-1} penicillin G, 6.13 mg ml^{-1} polymyxin B, 5 mg ml^{-1} tetracycline and 1 mg ml^{-1} amphotericin B. The cysts were washed and set to hatch in filter-sterilised tap water. An overnight hatch of J2s was counted and sterilised sequentially for 5 min periods with each of the following antibiotics; 0.1 % streptomycin sulphate, 0.1 % penicillin G, 0.1 % amphotericin B and 0.1 % cetyltrimethylammoniumbromide; Cetrimide (Sigma Chemical Co., Dorset, U.K.). J2s were collected by microcentrifugation for 10 seconds between treatments and were finally washed extensively in filter sterilised tap water before use.

Sterilised juveniles were inoculated onto root tips of transformed *Arabidopsis* seedlings as described for *M. incognita supra*. Plants were removed from the agar at 2 day intervals until 14 days after infection and then at 21 and 28 days after infection. Root systems were stained and examined as for infections with *M. incognita (supra)*.

Results:

Arabidopsis plants transformed with the $PsMT_A$ promoter:GUS construct and infected with *H. schachtii* exhibited strong expression throughout the root system and around the site of infection of the nematode until 14 days after infection. Figure 5 shows the results of *A. thaliana* transformed with $PsMT_A$:GUS construct and infected with

Heterodera schachtii. The *A. thaliana* were stained for GUS activity at : A) 2 days post infection; B) 6 days post infection; C) 6 days post infection and D) 8 days post infection. The nematode is indicated with an arrow in each case.(See Figure 5). By 21 days after infection there was some localised down-regulation of the promoter around the site of nematode infection.

Example 3: Expression of the engineered oryzacystatin (OC1ΔD86) regulated by the TUB-1 promoter

DNA preparation and manipulation: as for Example 1.

The GUS gene was removed from the commercially available plasmid PBI121 (Clontech) as a *Bam*HI-*Sst*I fragment. A synthetic oligonucleotide linker was ligated into the cut vector such that the *Bam*HI and *Sst*I sites were recreated, and an additional *Kpn*I site was introduced between them.

The resulting plasmid was digested with *Hind*III and *Bam*Hi to remove the CaMV35S promoter which was directly replaced by the TUB-1 promoter, also as a *Hind*III-*Bam*HI fragment. The coding region of the engineered oryzacystatin gene (OC1ΔD86) was inserted into the plasmid behind the TUB-1 promoter as a *Bam*HI-*Kpn*I fragment.

The final construct was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation, as in Example 2. The plasmid-containing bacteria were used to transform *Arabidopsis thaliana* C24, as in Example 2.

Example 4: Extending TUB-1 promoter sequences

The 560 bp fragment of the TUB-1 promoter which was used to make the TUB-1:GUS construct described in Example 1 was identified as too short to confer suitable expression in transgenic *Arabidopsis* (Leu *et al.*, *The Plant Cell*, 7:2187-2196 (1995) and our own observations). However, the fact that it was capable of directing GUS expression in transgenic tomato hairy roots and transgenic potato shows that the 560 bp TUB-1 promoter fragment is useful in some crop species. An inverse PCR technique was used to clone longer fragments of the TUB-1 promoter for use in other crop plants to provide root-specific expression.

Method for obtaining extended TUB-1 promoter sequences

1 µg of *Arabidopsis thaliana* C24 DNA, prepared as described in Example 1, was digested with BAMHI and the reaction mix extracted with phenol/chloroform and precipitated with ethanol following the addition of 0.1 volumes 3M sodium acetate pH 4.8. The precipitated DNA was self-ligated overnight at 16 °C and the ligation reaction was then used as a template for PCR. The primers used in the amplification were:

5' CGTAATGAATACAGTAACTTTGC 3'

and

5' CAAGAACTCATCCTACTTTGTTG 3'

Reaction conditions for PCR were as described in Example 1. Electrophoresis of the PCR products on an agarose gel revealed a single DNA band of 400 bp which was isolated from the gel by electroelution and cloned into the pCRII vector (Invitrogen). The DNA insert was completely

sequenced on both strands and this enabled the design of a further oligonucleotide primer which could be used with an existing primer to amplify a longer region of the TUB-1 promoter consisting of approx. 920 bp of upstream sequence. The sequence of this primer, designated TUB900 was:

5' ACAAAGCTTTACAAGTTCAATTATTG 3'

It was used in conjunction with the primer previously described in Example 1:

5' ACTATGGATCCGATCGATGAAGATTTTGGG 3'

in a PCR reaction comprising 7.5 ng *Arabidopsis* genomic DNA as described previously in Example 1. The PCR products were digested with Bam HI and HindIII, electrophoresed through an agarose gel, purified by electroelution and cloned into the plasmid vector pUC19 as described previously. The DNA insert was sequenced and confirmed as an extended fragment of the TUB-1 promoter (see Figure 6). The approximately 900 bp fragment was then cloned into the vector pBI101 as before. The approach can be used to extend the known sequence of the TUB-1 upstream region even further if a longer promoter fragment proves necessary for any crop species. The approach can be used to isolate promoter regions of any gene providing root-specific expression if unknown additional upstream sequence is needed to ensure the specific pattern of expression required.

Example 5: Construct of the TUB-1 promoter and the anti-nematode protein modified oryzacystatin

In this example, the 560 bp TUB-1 promoter fragment, from Example 1 was cloned into a plant transformation vector in conjunction with a modified plant cysteine proteinase inhibitor (cystatin). This work was carried out to demonstrate that the promoter can deliver biologically active expression levels of an anti-nematode protein using a cystatin as a specific example.

DNA preparation and manipulation

As for Example 1.

Preparation of the TUB-1:OcIΔD86 construct

The commercially available plasmid pBI121 (Clontech) consists of the GUS gene under the control of the CaMV35S promoter. The GUS gene was removed from this plasmid as a BamHI-Sst I fragment and replaced with a synthetic oligonucleotide linker which recreated the BamHI and SstI sites and introduced an additional KpnI site between them.

The resulting plasmid was digested with HindIII and BamHI to remove the CaMV35S promoter and this was directly replaced by the TUB-1 promoter, also as a HindIII-BamHI fragment. The oryzacystatin gene, Oc-I, has been modified to produce a variant (Oc-IΔD86) which has a greater detrimental effect on the growth and development of nematodes (Urwin *et al.*, *The Plant Journal*, **8**:121-131 (1995)). This modified gene was cloned as a BamHI-KpnI fragment into the plant transformation vector containing the TUB-1 promoter.

The resulting construct was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described for Example 1. The construct was introduced

into potato according to the method of Dale & Hampson (*Euphytica*, **85**:101-108 (1995)) and initial analysis of the Oc-IΔD86 content of leaf and root tissue has been carried out for a number of plants.

Determination of Oc-IΔD86 levels in transgenic potato plants.

Samples of potato root or leaf tissue were ground to a fine powder in liquid nitrogen and resuspended in PBS buffer supplemented with 2.5 μM trans-Epoxysuccinyl-L-Leucylamido(4-Guanido)-butane (E64) at levels that were somewhat more than required to inhibit native proteinases without sufficient excess to bind to all papain in the plate wells in the later assay. This level is found empirically for different plant tissues by increasing E64 concentrations in preliminary ELISA assays until further addition does not enhance detection of added Oc-IΔD86 in the range 0-1 % total soluble protein (tsp). Aliquots of protein extract were added to the wells of a microtitre plate previously coated with papain (10 μg/well) to capture the Oc-IΔD86. This was then quantified by a standard two-antibody sandwich ELISA (Harlow & Lane, Antibodies - A laboratory manual, Cold Spring Harbor, New York (1988)) using a polyclonal antibody raised against Oc-I and an alkaline phosphatase conjugated rabbit anti-rat secondary antibody diluted 1 in 2,000. Alkaline phosphatase activity was measured by monitoring p-nitrophenyl phophate hydrolysis at 405 nm. Non-transformed potato extract spiked with purified recombinant Oc-IΔD86 (0-1 % tsp) was used to construct a standard curve. Potato plants transformed with a CaMV35S:Oc-IΔD86 construct were analysed in the same way for comparison.

Results

As expected, the constitutive promoter CaMV35S directed expression of Oc-IΔD86 in both leaf and root tissue of transformed potato plants. In contrast, the TUB-1 promoter provided similar expression levels in roots but no detectable level in leaves (see Table). In all cases, values were compared with values for the corresponding tissue of untransformed potato plants. The expression of an anti-nematode protein, in this case a proteinase inhibitor, can therefore be restricted to root systems.

Construct promoter/effector	**Leaf (%tsp)**	**Root (%tsp)**
CaMV35S:Oc-IΔD86	0.058 ± 0.003**	0.096 ± 0.009***
TUB-1:Oc-IΔD86	0 ± 0.0007 NS	0.077 ± 0.003**

Table 1. Estimated expression levels as % of total soluble protein (% tsp) in leaf and root tissue of transformed potato plants for the effector protein Oc-IΔD86 given by two constructs differing only in promoters. Values are for example lines and estimates were provided by ELISA (see text for details). Values were compared using One-way ANOVA with *a priori* contrasts against corresponding untransformed tissue (NS, not significant P=0.5; **, P<0.01; ***, P<0.001).

The RPL16A gene from *Arabidopsis thaliana* encodes the ribosomal protein, L16. Transcription of the RPL16A promoter is cell specific and promoter:GUS fusions show it to be expressed in internal cell layers behind the root meristem, dividing pericycle cells of mature roots, lateral root primordia and the stele of developing

lateral roots. Expression was also observed in developing anthers and pollen (Williams & Sussex, *The Plant Journal*, **8**:65-76(1995)).

The ARSK1 gene from *Arabidopsis thaliana* encodes a protein with structural similarities to serine/threonine kinases. Its expression is root specific as judged from a promoter:GUS fusion construct reintroduced into *Arabidopsis*. There were high levels of expression in the epidermal, endoepidermal and cortex regions of the root (Hwang & Goodman, *The Plant Journal*, **8**:37-43 (1995)).

Example 6: Cloning of the RPL16A promoter

DNA preparation and manipulation

As for Example 1.

GUS expression directed by the RPL16A promoter

Genomic DNA was prepared from *Arabidopsis thaliana* as for Example 1. The RPL16A promoter region was amplified by PCR from the *Arabidopsis* genomic DNA using two oligonucleotide primers with the sequences:

5' ACAAAGCTTAACGAAAGCCATGTAATTTCTG 3'

and

5' ACAGGATCCCTTCAAATCCCTATTCACATTAC 3'

designed from the published sequence of the RPL16A upstream region (Williams & Sussex, *The Plant Journal*, **8**:

65-76 (1995)). Restriction enzyme sites HindIII and BamHI were incorporated into the primers to aid cloning of the amplified product. PCR amplification of the RPL16A promoter fragment was carried out as described in Example 1. The amplified DNA was digested with HindIII and BamHI and a specific DNA fragment was recovered from an agarose gel and cloned into the plasmid vector pUC19 (Yanisch-Perron *et al.*, (1985) infra). The sequence of the RPL16A promoter was verified (see Figure 7).

The RPL16A promoter was then introduced into the vector pBI101 (Clontech) as a HindIII/BamHI fragment.

Introduction of the construct into *Agrobacterium tumefaciens* LBA4404 and transformation of *Arabidopsis thaliana* with the RPL16A:GUS construct was as described for Example 2. Staining of roots with X-gluc was carried out as described for TUB-1 transformed hairy roots.

Results

Uninfected roots of *Arabidopsis* plants transformed with the RPL16A promoter:GUS construct showed expression particularly in lateral root primordia and internal cell layers just behind the root tip. Figure 8 shows the results of *A. thaliana* transformed with the RPL16A:GUS construct and stained for GUS activity. In the Figure A) GUS expression is evident in cells behind the root meristem and in developing vascular tissue and B) GUS expression occurs in a lateral root primordium.

Example 7: Cloning of the ARSK1 promoter

DNA preparation and manipulation

As for Example 1.

GUS expression directed by the ARSK1 promoter

A DNA fragment containing a region of the ARSK1 promoter was amplified from *Arabidopsis thaliana* genomic DNA by PCR as described in Example 1 using two oligonucleotide primers with the sequences:

5′ ACAAAGCTTATCTCATTCTCCTTCAAC 3′

and

5′ ACAGGATCCTTCAACTTCTTCTTTTG 3′

designed from the published sequence of the ARSK1 upstream region (Hwang & Goodman, *The Plant Journal*, 8:37-43 (1995) and GenBank Accession No. L22302).

The amplified DNA fragment was digested with HindIII and BamHI and cloned into the plasmid vector pUC19 as described in Example 1. The ARSK1 promoter was then introduced into the vector pBI101 (Clontech) as a HindIII/BamHI fragment (sequence shown in Figure 9). The construct was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation as for TUB-1 and this was then used to transform *Arabidopsis thaliana* C24 as described in Example 2.

Example 8: Manipulation of promoter regions to enhance specificity

This example describes how promoter deletions may be created to identify regions of the promoter which are essential for expression in roots and/or to manipulate a promoter to have greater root specificity. This example uses the promoter from the pea metallothionein-like gene, $PsMT_A$.

DNA Preparation and Manipulation

As for Example 1.

Preparation of deletion constructs

A total of 7 deletion constructs were created in the vector pBI101.2, designated $PsMT_A\Delta1$ (210 bp), $PsMT_A\Delta2$ (282 bp), $PsMT_A\Delta3$ (393 bp), $PsMT_A\Delta4$ (490 bp), $PsMT_A\Delta5$ 585 bp), $PsMT_A\Delta6$ (632 bp) and $PsMT_A\Delta7$ (764 bp).

For Δ1, Δ2, Δ5, Δ6, and Δ7 restriction sites were used to create the deletions, which were subcloned into pUC18 and then transferred to pBI101.2 as *Hind* III/*Bam* HI fragments. The extent of the deletions and the restriction sites used are indicated on Figure 10.

For the Δ3 and Δ4 constructs no suitable restriction sites were available so oligonucleotide primers were synthesized and used in PCR reactions to amplify the desired promoter regions. The primers for the Δ3 deletion were:

5' ATTTATTGAAACAAGTAATCATCC 3'

and

5' GGAAACAGCTATGACCATG 3' (M13 reverse primer)

The primers for the Δ4 deletion were:

5' TATTAAGCTTCCCGTGACATTATTAAATAC 3'

and

5' GGAAACAGCTATGACCATG 3' (M13 reverse primer)

The template for the PCR reaction in each case was a pUC18 plasmid clone containing the complete $PsMT_A$ promoter region as a *Hind* III/*Bam* HI fragment. Conditions for the PCR reaction were as described in Example 1. The amplified fragment from the Δ3 PCR was cloned directly into pCRII (Invitrogen) and verified by sequencing. A *Hind* III/*Bam* HI fragment containing the deleted promoter was then cloned into pBI101.2.

The product of the Δ4 PCR was digested with *Hind* III/*Bam* HI, cloned first into pUC18, and from there into pBI101.2.

Constructs were introduced into *Agrobacterium tumefaciens* as in Example 1 and have been used to transform *Arabidopsis*.

Results

Transformants have been recovered for the Δ2, Δ5 and Δ6 deletion reporter constructs. When stained with X-gluc to reveal GUS activity as described in Example 1, the Δ5 and Δ6 plants showed an identical pattern of expression to plants transformed with the full length promoter construct. In contrast, plants transformed with the Δ2 construct displayed no GUS activity in roots but only in leaf hydathodes, and some flower parts. This implies that a region between -585 and -282 bp must be

responsible for expression in root tissue. The Δ3 and Δ4 constructs should define more precisely the role of this region of DNA and it may then be possible to use this information to create a promoter construct which has only activity in roots.

CLAIMS:

1. Nucleic acid comprising a transcription initiation region capable of directing expression predominantly in the roots of a plant, and a sequence which encodes an anti-nematode protein.

2. Nucleic acid as claimed in claim 1 wherein the transcription initiation region is a promoter.

3. Nucleic acid as claimed in claim 2 wherein the promoter is the promoter from the b1-tubulin gene of *Arabidopsis* (TUB-1) or the promoter from the metallothionein-like gene of *Pisum sativum* ($PsMT_A$).

4. Nucleic acid as claimed in any one of claims 1 to 3 which also comprises a transcription termination sequence.

5. Nucleic acid as claimed in any one of claims 1 to 4 wherein the anti-nematode protein is effective against one or more of the following nematode genera, *Heterodera, Globodera, Meloidogyne, Hoplolaimus, Helicotylenchus, Rotylenchoides, Belonolaimus, Paratylenchus, Paratylenchoides, Radopholus, Hirschmanniella, Naccobus, Rotylenchulus, Tylenchulus, Hemicycliophora, Criconemoides, Criconema, Paratylenchus, Trichodorus, Paratrichodorus, Longidorus, Paralongidorus* or *Xiphinema*.

6. Nucleic acid as claimed in claim 5 wherein the anti-nematode protein is effective against one or more of the following nematodes, *Meloidogyne incognita, M. javanica, Globodera pallida, G. rostochiensis,*

Heterodera schachtii, *Heterodera glycines*, *M. arenaria* or *M. hapla*.

7. Nucleic acid as claimed in any one of claims 1 to 6 wherein the transcription initiation region is one which undergoes up-regulation at a nematode infected location.

8. Nucleic acid as claimed in any one of claims 1 to 7 wherein the anti-nematode protein is a collagenase, a lectin, an antibody, a toxin of *Bacillus thuringiensis* or a proteinase inhibitor.

9. Nucleic acid as claimed in claim 8 wherein the protein is a cystatin.

10. Nucleic acid as claimed in claim 9 wherein the cystatin is oryzacystatin 1, having amino acid 86 deleted (or OC1ΔD86).

11. Nucleic acid as claimed in any one of claims 1 to 10 which is in the form of a vector.

12. The use of nucleic acid comprising a transcription initiation region capable of directing expression predominantly in the roots of a plant, in the preparation of a nucleic acid construct adapted to express an anti-nematode protein.

13. The use as claimed in claim 12 modified by any on or more of the features of any one of claims 2 to 11.

14. A method of preparing nucleic acid as defined in any one of claims 1 to 11 which comprises coupling

together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides.

15. A method as claimed in claim 14 wherein the nucleic acid is prepared recombinantly.

16. A method of conferring nematode resistance on a plant which comprises the step of transforming the plant with nucleic acid as defined in any one of claims 1 to 11.

17. The use of nucleic acid as defined in any one of claims 1 to 11 in the preparation of a transgenic plant having nematode resistance.

18. A plant cell transformed with nucleic acid as defined in any one of claims 1 to 11.

19. A plant comprising plant cells as defined in claim 18.

20. A process for the manipulation of a transcription initiation region to alter the specificity of the transcription initiation region.

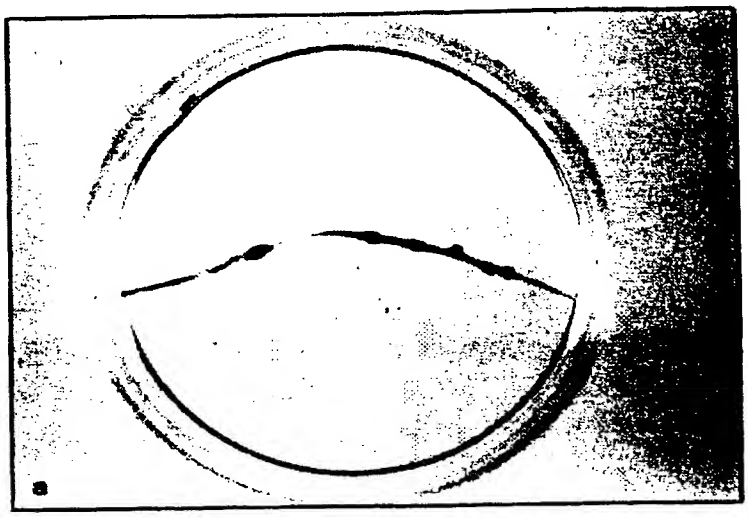
Sequence of TUB-1 promoter

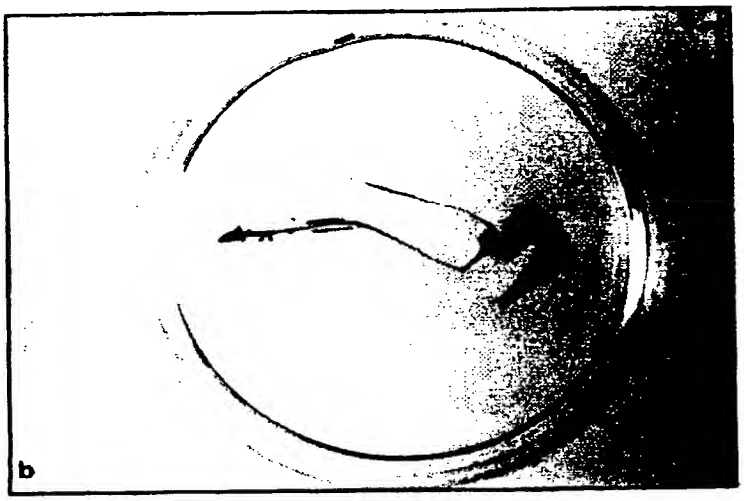
AAGCTTGTT ACTGTATTCA TTACGCATTG ATCGTCTTCT TCTACGCACT
ATTATTGAAT TTTTGTTTCT CGATGTTTTC AGAAGTCATA ACATATAATC
AAGGGTGGTT GTAGCTAGTC GTGATGAAAT TCGAATAGTA AAAGATTCAT
CAAATGGATC AAGATTTGAA ACTAGAATGA ATGATCGATA CTTGAAAGCT
ATCACACTTA AATAGTTAAA TGGACGATAT AATTCAACAA TATCTTAAAT
AACATTAATC ATTCACTTTG TAGAAATTTC AATTTGACGG TGCTAACAAC
AATATGTGAC ATTTTTGTTT GCAGCAAAAA AGTCAAATGG TAAAAAAAAA
CATTTTTTTA AAAAGAGAGT ATAGTAAAAA AAACAGAGGA TAAAAAACGA
ACAAACGGTT AAAGCTGGCA AAGATCCCAT CTTCCCCACG CTGTCAAATT
TTGTCACTAC TTCCCAAAAA AAACTCTCCC GCCTCTCTCT CTCCCTCTCC
CTTCTTATTC TCCTCCTTCT TCTCCTTCAC CATCTCCAGA TCCCAAAATC
TTCATCGATC GGATCC

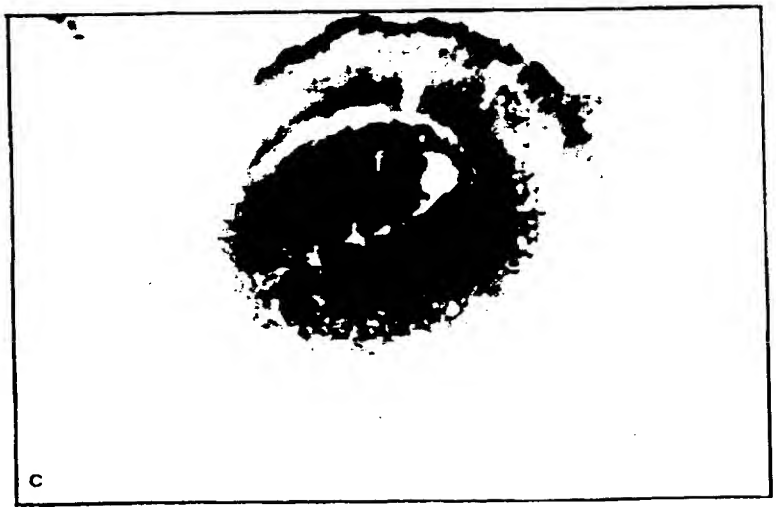
The *Hin*dIII and *Bam*HI restriction sites introduced with the PCR primers are included in this sequence.

FIG. 1

FIG. 2







Sequence of $PsMT_A$ promoter region introduced into pBI101.2

AACCAAGTTT CTTGTTCTTC AAACATTTTC TAATTCTTCA GCTTAGAGTT

TCCTTTCATA ATTAATGTAA GAATTGACCC TAGTAAGAAA GTCACTCATA

CCCTTTCCTT CTTCTAGCCC TAAATTTCCG TGAAACATAT AGTCTTCCCT

TAACCCTTTT TGGAACATCC AACATTGCAG CTTATCATAT ATTGATCCTA

CTTTGGTGAA GCGTTATATG TACTCCCGGA GGATTTCCAT TTTCATCTAT

TGGATTCCTA TGAAGACAAC TATAGTCTTG GATTTCAATT TTTTAATTAA

TCAAAAATAA ATTATCCCGT GACATTATTA AATACCATTT TGAAATAAAA

AGTTTCTAAA AAAAGTCCTT CGTCATTATC CATCATTTTT ATTAAAGTAT

ATTTTATTAT TTATTGAAAC AAGTAATCAT CCATTAGCGG AGAAATAGAG

GAGTGAAGCA TTTAACTTTT CCAAACGAAA GCGACGTAAT CAACCTACAT

TTGACTTAGA TTGGATTAAG CATGCAACAA ATTAAAATTT AATCGCCATT

GCAATTTGCA CACCACAATA AGACGTGTGA TGAAAACGAT GATATCTACG

TGGAAATAAT CCAAGGGTGG CCTTGTGGAC CCATGCAACA CAGGATGACA

ACACGTGGAC GGTCAAGATT TCACCAATTA TTCTCTCCCA CCTTATAAAT

TGGGGCACGC AACATCATTA AAAGACATCA ATTGTAGTGA AGATAACAGC

AACCAAGCAA TTAATATCAA TTGTTGTTTG CAAAAAATCT TAGGTTCTGA

AAAT **ATG TCT GGA TGT GGT TGT GGG** GAT CC

This sequence includes the first 7 codons of the *$PsMT_A$* coding sequence (shown in bold) and the introduced *Bam*HI site which allows a translational fusion to be made between $PsMT_A$ and GUS in pBI101.2.

FIG. 3

FIG. 4

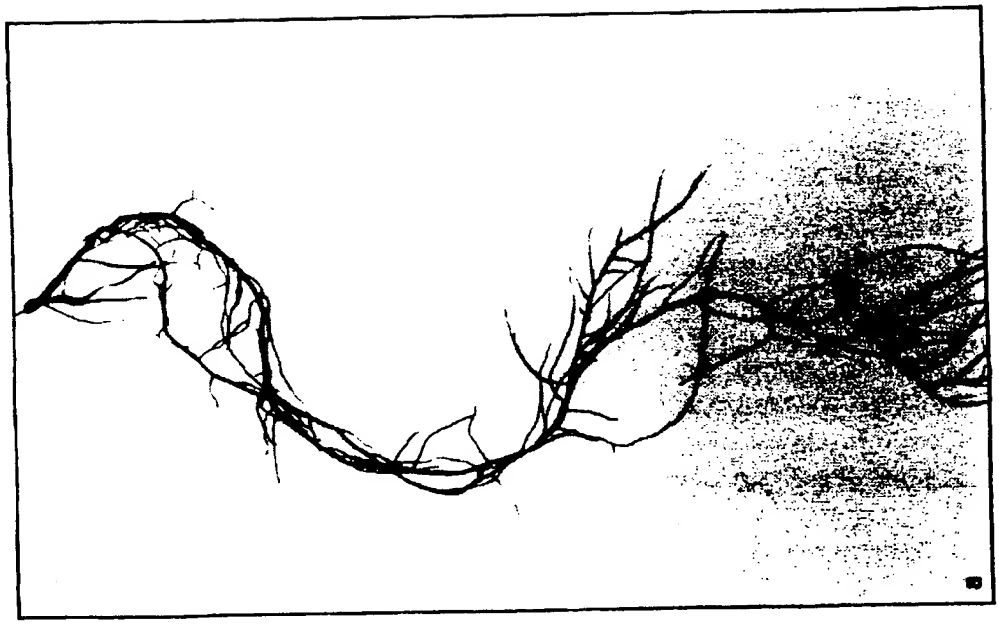
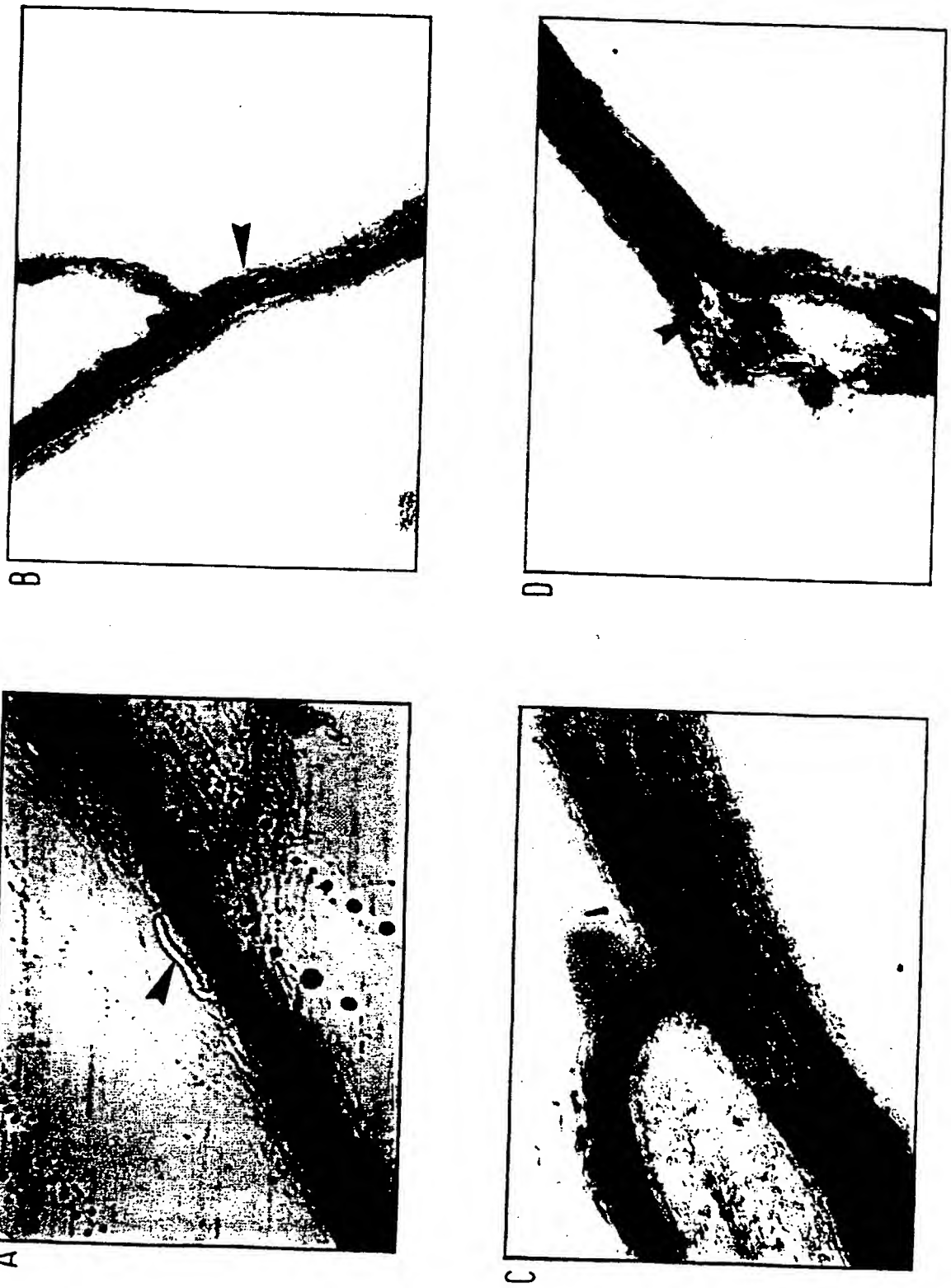


FIG.5



```
1     TTTACAAGTT CAATTATTGA TTTGGACGTT ACGTATAAAC AGTAGATCGA CCATGACATA
61    AATAAAAATG AAAAAATAAT TGGATCAAAT CTCAGATCTT ACTACAGTCT ACATCTACCT
121   AGCTACATGT GCTATGGCAT TATTTGTTTT TCATGTTATC ATCAATATTG TATAATAGAT
181   CCAAACACAA TCAGTGTGAT GCTATTTTTC TATACTTTTG ATCATTCAAC CGATTTCAAT
241   TATTGTGTTT TGTCGTTTAC ATAGAAATAG GAACATATGC ATTCAAGATC TCCAGATCGT
301   TTATTACGTC TTGCTACATG TGCTATGTCA CTAAAACAAC TAAGATACTA CATGCAAAGT
361   TACTGTATTC ATTACGCATT GATCGTCTTC TTCTACGCAC TATTATTGAA TTTTTGTTTC
421   TCGATGTTTT CAGAAGTCAT AACATATAAT CAAGGGTGGT TGTAGCTAGT CGTGATGAAA
481   TTCGAATAGT AAAAGATTCA TCAAATGGAT CAAGATTTGA AACTAGAATG AATGATCGAT
541   ACTTGAAAGC TATCACACTT AAATAGTTAA ATGGACGATA TAATTCAACA ATATCTTAAA
601   TAACATTAAT CATTCACTTT GTAGAAATTT CAATTTGACG GTGCTAACAA CAATATGTGA
661   CATTTTTGTT TGCAGCAAAA AAGTCAAATG GTAAAAAAAA ACATTTTTTT AAAAAGAGAG
721   TATAGTAAAA AAAACAGAGG ATAAAAAACG AACAAACGGT TAAAGCTGGC AAAGATCCCA
781   TCTTCCCCAC GCTGTCAAAT TTTGTCACTA CTTCCCAAAA AAAACTCTCC CGCCTCTCTC
841   TCTCCCTCTC CCTTCTTATT CTCCTCCTTC TTCTCCTTCA CCATCTCCAG ATCCCAAAAT
901   CTTCATCGAT C
```

FIG. 6

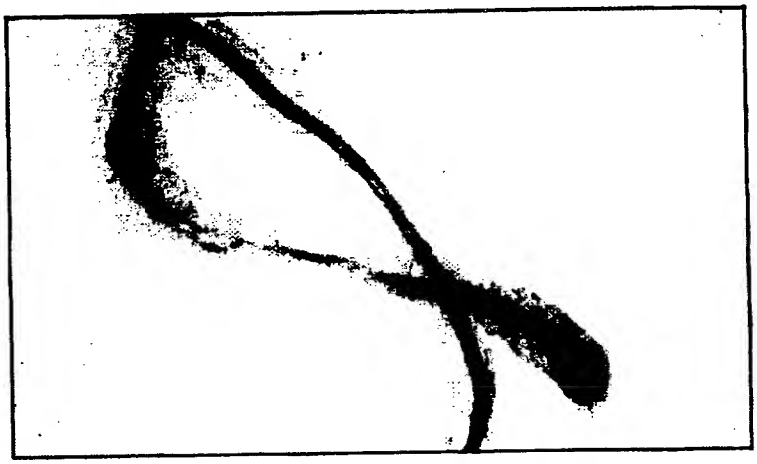
Sequence of the extended TUB-1 promoter

```
1   AACGAAAGCC ATGTAATTTC TGTGAATCTC AATCCGGTTT TGCTTAGTCT TAGGCACAGA
61  TTGACGATCA TCAATATCAA GATTGGAAAC CAAAACAAAC ATGTTCTTCC ACCTCTTAGA
121 GAGTACTGAG GTTGAAACAG CTTCTTTTGT GGAGATAAGA GACAGAATAC GCCCCAAGAA
181 CCTCATCTGG TAGACGGCTG ATTACATCTT TAGCATTCAT TGATACCAAA ACCTTAAAAC
241 CACAGCAATT GGATTCAGAA ACAGTAACTA GACAGAGTTG ATGAGTTCAA AGGTCTGTTC
301 TTTTCATACA ATGCAAAGTC TAATACAAAA GTTATCATGT ATCTCTTCAA TCACATTGAT
361 TACCATAGAA ATGAAAAAGA CGAAACAGAG CAGACAACAT ACCTTGCTAA AGTGCTACTT
421 GCTACAGAGT CGTGAAAGCT CTCCACTTTC AATACCAATT TAATTCGTGA GATCAAAAGT
481 GAAAACATCG AATACTAGGG TTTTTGGAAA GTTTAAGAGA AGAGACAAGA AACTGAAACA
541 ACTTATTATA AGCAATTTCC TCCATTTCCA CATGAGGAAA GAGTAAAGCA GAAAGACTGA
601 TGAAGAAAAG GTAAGTAGTC TGTTTGGGAA AGAAAGTCAC GTGAGAGAAG TCGTGGGCCT
661 TTAAGGGTAA AACTGTCATT TAAATAGTCG CCGTTCTTTA CACTTAGGAT TAGGGCTTCT
721 AACATTTAAA TAGTCGTTTC GTTCTCCGTC TCTCCTTTCC CGTCGGCCGG TGATAATTTC
781 GCAACCCAAA TTCCAAATGG TAAGTTTCAG TTCTTGGATT TGTATGATTT TGTAATGTGA
841 ATAGGGATTT GAAG
```

FIG. 7

Sequence of the *Arabidopsis thaliana* RPL16A promoter region cloned into pBI101.

A



B

FIG. 8

```
1    ATCTCATTCT CCTTCAACAA GGCGAATCAA ATCTTCTTTA TACGTAATAT
51   TTATTTGCCA GCCTGAAATG TATACCAAAT CATTTTTAAA TTAATTGCCT
101  AAATTATTAG AACAAAAACT ATTAGTAAAT AACTAATTAG TCTTATGAAA
151  CTAGAAATCG AGATAGTGGA ATATAGAGAG ACACCATTAA ATTCACAAAA
201  TCATTTTTAA ATTACCTAAA TTATTACAAC AAAAACTATT AGACAGAACT
251  AAGTCTATAA TGAAACGAGA GATCGTATTT GGAATGTAGA GCGAGAGACA
301  ATTTTCAATT CATTGATATA TAAGCAAAAT TATATAGCCC GTAGACTTTG
351  GTGAGATGAA GTCTAAGTAC AAACAACTGA ATGAATTTAT AATCAATAAT
401  ATTGATTATA TTGTGTTAGA AAAAGAAAAC AACTTGCGTT ATTTTTCAAT
451  ATTATTGTGA GGATTAATGT GAACATGGAA TCGTGTTTCT CCTGAAAAAA
501  ATATCAGCAT AGAGATTAGA ACAATATAAA TATATCCACC AAAAATAACT
551  TCAACATTTT TATACAACTA ATACAAAAAA AAAAAAGCAA ACTTTTTGTA
601  TATATAAATA AATTTGAAAA CTCAAAGGTC GGTCAGTACG AATAAGACAC
651  AACAACTACT ATAAATTAGA GGACTTTGAA GACAAGTAGG TTAACTAGAA
701  CATCCTTAAT TTCTAAACCT ACGCACTCTA CAAAAGATTC ATCAAAAGGA
751  GTAAAAGACT AACTTTCTCC ATTTTTTCCC CAAATAACAC AAAAGAAGAA
801  GTTGAA
```

Sequence of the *Arabidopsis thaliana* ARSK1 promoter region cloned into pBI101.

FIG. 9

⇓ Δ7

AACCAAGTTT CTTGTTCTTC AAACATTTTC TAATTCTTCA GCTTAGAGTT

TCCTTTCATA ATTAATGTAA GAATTGACCC TAGTAAGAAA GTCACTCATA

CCCTTTCCTT CTTCTAGCCC TAAATTTCCG TGAAACATAT AGTCTTCCCT

⇓Δ6

TAACCCTTTT TGGAACATCC AACATTGCAG CTTATCATAT ATTGATCCTA

⇓Δ5

CTTTGGTGAA GCGTTATATG TACTCCCGGA GGATTTCCAT TTTCATCTAT

TGGATTCCTA TGAAGACAAC TATAGTCTTG GATTTCAATT TTTTAATTAA

⇓Δ4

TCAAAAATAA ATTATCCCGT GACATTATTA AATACCATTT TGAAATAAAA

AGTTTCTAAA AAAAGTCCTT CGTCATTATC CATCATTTTT ATTAAAGTAT

⇓Δ3

ATTTTATTAT TTATTGAAAC AAGTAATCAT CCATTAGCGG AGAAATAGAG

GAGTGAAGCA TTTAACTTTT CCAAACGAAA GCGACGTAAT CAACCTACAT

⇓Δ2

TTGACTTAGA TTGGATTAAG CATGCAACAA ATTAAAATTT AATCGCCATT

⇓Δ1

GCAATTTGCA CACCACAATA AGACGTGTGA TGAAAACGAT GATATCTACG

TGGAAATAAT CCAAGGGTGG CCTTGTGGAC CCATGCAACA CAGGATGACA

ACACGTGGAC GGTCAAGATT TCACCAATTA TTCTCTCCCA CCTTATAAAT

TGGGGCACGC AACATCATTA AAAGACATCA ATTGTAGTGA AGATAACAGC

AACCAAGCAA TTAATATCAA TTGTTGTTTG CAAAAAATCT TAGGTTCTGA

AAATATGTCT GGATGTGGTT GTGGGGATCC

$PsMT_A$ promoter region showing the extent of deleted promoter constructs which have been created. The arrow (⇓) indicates the first deleted nucleotide with all those 5' to it deleted. A series of deletions (Δ1-Δ7) where made.
The initial part of the coding region which is included in the constructs is underlined.

FIG. 10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02942

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/29 C12N5/10 A01H5/00 A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H A01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 12015 A (CHUA NAM HAI) 9 June 1994 see page 20, line 15 - page 21, line 2 ---	1,20
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X	WO 93 06710 A (UNIV NORTH CAROLINA) 15 April 1993 see page 20, line 16 - line 34 see page 21 - page 26 --- -/--	1,2,4-8, 11-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 April 1997	09. 04. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Maddox, A

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International Application No
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